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April 6, 1999

Commissioner of Patents and Trademarks
U S Patent and Trademark Office
Washington, DC 20231

Dear Sir

RE.   New US Patent Application Entitled: **BRCA1 AND hMLHI GENE PRIMER SEQUENCES AND METHOD FOR TESTING**
Inventor Jan Vijg, of San Antonio, Texas
Corresponding to Provisional Application U.S.Serial No. 60/084408, May 6, 1998

We enclose the above-identified patent application, together with 2 (two) sheets of drawings, and a Verified Statement (small entity status). Declaration (Power of Attorney) will follow shortly

The Commissioner is hereby authorized to charge the filing fee and any additional fees that may be due, to the deposit account No. 18-1425 of the undersigned attorney,

Cordially,

RINES AND RINES

Robert H Rines

RHR/ipo--Enclosures

Applicant or Patentee: Jan Vijg   Attorney's
Serial or Patent No.:   Corresponding to Provisional US Application   Docket No.: _____
Filed or Issued: No. 60/084408, May 6, 1998
For: BRCA1 and hMLHI Gene Primer Sequences And Method For Testing

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9(f) and 1.27(b)) - <u>INDEPENDENT INVENTOR</u>

As a below named inventor, I hereby declare that I qualify as an independent inventor
as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under section 41(a)
and (b) of Title 35, United States Code, to the Patent and Trademark Office with
regard to the invention entitled .   as above
described in

    [ ]   the specification filed herewith   60/084408
    [x]   application serial no. (Provisional)/_____, filed   May 6, 1998
    [ ]   patent no. _____, issued _____.

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I acknowledge the duty to file, in this application or patent, notification of any
change in status resulting in loss of entitlement to small entity status prior to
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due after the date on which status as a small entity is no longer appropriate. (37 CFR
1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that
all statements made on information and belief are believed to be true; and further
that these statements were made with the knowledge that willful false statements and
the like so made are punishable by fine or imprisonment, or both, under section 1001
of Title 18 of the United States Code, and that such willful false statements may
jeopardize the validity of the application, any patent issuing thereon, or any patent
to which this verified statement is directed.

Jan Vijg
NAME OF INVENTOR   NAME OF INVENTOR   NAME OF INVENTOR

Robert H. Rines, Esq. /Robert H Rines/
Signature of Inventor Counsel   Signature of Inventor   Signature of Inventor

May 6, 1999
Date   Date   Date

BRCAI AND hMLHI GENE PRIMER SEQUENCES
AND METHOD FOR TESTING

The present application is based upon provisional application 60/084408, filed

May 6, 1998, and is directed to methods of and primer sequences for sequence varia-

tion and/or mutation detection of BRCA and hMLH1 genes , such as by two-

dimensional denaturing gradient electrophoresis techniques (TDGS).

Background

Such techniques are described in Method Of And Apparatus For

Diagnostic DNA Testing, Jan Vijg and Daizong Li, PCT/IB96/00543, filed

3 June 1996, International Publication Number WO96/39535, 12 December 1996,

and in "Two-Dimensional DNA Typing", Molecular Bio Technolgoy, Vol. 4, 1995,

pp 275-295

The tests leading to the establishment of the primer sequences for the BRCAI

and hMLH1 of the present invention were conducted with the TDGS design prepared

with the computer programming and equipment described in PCT/IB97/00976, pub-

lished on or about February 14, 1998.

Objects of Invention and Summary

The objects of the invention are to provide novel theoretically and empirically

(experimentally) derived TDGS patterns for hMLH1 and BRCA1 genes which may be

used by testers to test for gene sequence variation and/or mutations.

<u>Drawings</u>

Figs. 1A and 1B show the computer-aided design TDGS patterns obtained for the hMLH1 and BRCA1 (theoretical-left hand side, empirical or experimental--right hand side)

In the theoretical vs. empirical patterns of the MLHI and BRCA1 genes, for all four genes, one or more exons were designed in overlapping fragments, in which case the fragment name is exon.1, exon..2, etc. Exons 8 and 15 of hMLH1 contain polymorphisms, which can be distinguished from disease-causing heterozygous mutations on the basis of a unique four-spot pattern (18).

<u>Description Of The Invention In Preferred Forms</u>

The MLH1 DNA mismatch repair gene. The design for *MLH1* took 30 minutes (excluding exon indication). Fig. 1A shows the theoretical and the empirical TDGS pattern for the *MLH1* gene. Because exons 11 and 12 had to be subdivided into overlapping fragments, two multiplex groups are currently being used, with the long PCR carried out as a four-plex PCR. Like many other genes, exon 1 of *MLH1* is GC-rich and, hence, was found to melt at a much higher % UF compared to most of the other fragments. Thus far, a total of 41 coded samples with previously identified mutations have been analyzed in a blinded fashion with 100% concordance (30).

The breast and ovarian cancer susceptibility gene BRCA1. The tumor suppressor gene *BRCA1* contains 24 exons, of which exon 11 contains approximately 60 % of the coding region. Fig. 1B shows the theoretical and empirical 2-D pattern for *BRCA1*. Of all 2-D designs discussed, this was the most difficult (total design time was 2 h), the main reason being the need to make overlapping fragments for the 3.4 kb exon 11. Pre-amplification was accomplished by one 7-plex long PCR. Using the long PCR amplicons as template, all 24 exons were amplified in a total of 37 fragments distributed over 5 multiplex groups. The overlap and sometimes short distances from fragment to fragment necessitated the use of so many multiplex short PCR groups. The non-coding exons 1a, 1b and the non-coding part of exon 24 were excluded. Evaluation of this test design using a panel of coded samples with previously identified mutations is currently ongoing. Thus far, mutations and polymorphisms have been detected in exons 2, 8, 11, 16, 20 and 23.

PCR Amplification

Primers were obtained from Genosys Biotechnologies, Inc. (The Woodlands, TX). For complete lists of all sequences, except BRCA1, see references 18, 29 and 30. Primer sequences for BRCA1 will be published elsewhere but will be made available upon request. PCR amplification of gene sequences was carried out using the two-step protocol first described by Li and Vijg (22). Primers for long-distance PCR were designed based on published sequences (24-27) using Primer Designer 3, to amplify the entire gene-coding region for each of the 4 genes as a 1-plex (TP53), a 6-plex PCR (RB1), a 4-plex PCR (MLH1) or a 7-plex PCR (BRCA1). The LA PCR kit (Takara) was used for long PCR in a PTC-100 thermocycler (MJ Research). Multiplex short PCR was carried out using the long PCR products as template. Between 0.1 and 1.125 µM of each primer was used in a 50 µl reaction with 1 µl of long PCR product in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 250 µM of each dNTP and 5 % formamide. Two and a half units of Taq DNA polymerase (Life Technologies) were added after an initial denaturation at 94 °C for 60 s. Cycling conditions for multiplex short PCR and concentrations of $MgCl_2$ varied among different genes and amplifications were carried out in a PTC-100 thermocycler (MJ Research).

Two-dimensional DNA electrophoresis

For RB1, 5 µl of multiplex short PCR was used per electrophoresis run. For TP53, MLH1 and BRCA1, 5 µl of each of the different multiplex groups were combined. One tenth of a

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volume of loading buffer (0.25 % xylene cyanol. 0.25 % bromophenol blue, 15 % ficoll

and 100 mM Na₂EDTA) was added and the mixtures were loaded onto a 6.5 % (*TP53*) or

10 % (*RB1, MLH1* and *BRCA1*) PAA non-denaturing size gel (acrylamide: bisacrylamide

= 37.5:1) in 0.5 x TAE buffer. The samples were electrophoresed for 5.3 h at 150 V

(*RB1*), 5 h at 120 V (*TP53*) or 7.5 h at 140 V (*MLH1* and *BRCA1*) at 50 °C. After staining

the gel with a mixture of equal amounts of SYBR-green I and II (Molecular Probes,

Eugene, Oregon) for 20 min, the region containing all fragments of interest (usually

between 100 and 600 bp) was cut out and loaded onto a denaturing gradient gel (DGGE).

Gradients used were 0 to 50 % UF for *RB1*, 20 to 70 % UF for *TP53*, 25 to 70 % UF for

MLH1 and 20 to 65% UF for BRCA1. The second orthogonal dimension was run for 12

h at 100V (RBI), 14 h at 120 V (TP53) or 16 h at 100 V (MLH1 and BRCA1). Spot

patterns were visualized by SYBR-green staining using a FluorImager (Molecular

Dynamics, Sunnyvale, California).

What is claimed is:

1.   A method of enabling BRCA1 and h MLHI gene testing for gene sequence variation and/or mutations, that comprises, preparing appropriate test kit primers and solvents suitable for amplifying by long distance PCR the entire gene-coding region of each of BRCA1 and MLHI genes, to be followed by multiplex short PCR using the long PCR products as templates; providing in such test kit appropriate buffer and gel and solvent materials for use in electrophoresis in orthogonal dimensions for producing spot patterns indicative of gene sequence variations and/or mutations.

2.   The method of claim 1 wherein the test kit is provided with non-denaturing gel and buffer materials suitable to enable combined mixtures of the multiplex groups of BRCA1 and MLHI to be subjected to the electrophoresis simultaneously together.

3.   The method of claims wherein, the kits provide the Primer Pairs A and B listed in the specification for Long Distance and Short PCR, respectively.

4.   Test kits for enabling BRCA1 and h MLHI gene testing prepared by the method of claim 3.

ABSTRACT

Primer sequences and materials are pre-prepared as test kits for enabling

appropriate gene scanning patterns, preferably by two dimensional electrophoresis

(TDGS), for use in detecting sequence variations and/or mutations in BRCA1 and h

MLH1 genes

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6 The primer sequences for long and short PCR for the BRCA1 are as

follows:

A. Primer Pairs for Long Distance PCR

Exons 1-4

   MLH1-4F GCG.GCT.AAG.CTA.CAG.CTG.AAG.GAA.GAA.CGT.GA

   MLH1-4R GGC.GAG.ACA.GGA.TTA.CTC.TGA.GAC.CTA.GGC.CC

product size= 10.8kb

Exons 5-10

   MLH5-10F

GCG.CCC.CTT.GGG.ATT.AGT.ATC.TAT.CTC.TCT.ACT.GG

   MLH5-10R GCG.CTC.ATC.TCT.TTC.AAA.GAG.GAG.AGC.CTG

product size=10.5kb

Exons 11-13

   MLH11-13F CGG.CTT.TTT.CTC.CCC.CTC.CCA.CTA.TCT.AAG.G

   MLH11-13R GGG.TTA.GTA.AAG.GAA.GAG.GAG.CTT.GCC.C

product size=8.7kb

Exons 14-19

   MLH14-19F GGT.GCT.TTG.GTC.AAT.GAA.GTG.GGG.TTG.GTA.G

   MLH14-19R

GCG.CGC.GTA.TGT.TGG.TAC.ACT.TTG.TAT.ATC.ACA.C

product size=10.5kb

Underlined nucleotides represent nucleotides added to modify melting temperatures

of the primers

B. Primer Pairs for Short PCR

Exon Clamp[1] Product Size Tm[2] Primer Sequence

12.1	40	184	44.53	TTT.TTT.TTT.TTT.TAA.TAC.A AAT.CTG.TAC.GAA.CCA.TCT
12.2	8 40	366	53.23	TGG.AAG.TAG.TGA.TAA.GGT TGT.ACT.TTT.CCC.AAA.AGG
13	40	272	49.06	ATC.TGC.ACT.TCC.TTT.TCT AAA.ACC.TTG.GCA.GTT.GAG
14	45 5	235	48.94	TAC.TTA.CCT.GTT.TTT.TGG GTA.GTA.GCT.CTG.CTT.GTT
15	40	179	29.97	CAG.CTT.TTC.CTT.AAA.GTC CAG.TTG.AAA.TGT.CAG.AAG
16	40	261	47.56	CTT.GCT.CCT.TCA.TGT.TCT.TG AGA.AGT.ATA.AGA.ATG.GCT.GTC
17	40	199	47.01	ATT.ATT.TCT.TGT.TCC.CTT AAT.GCT.TAG.TAT.CTG.CCT
18	45	215	46.67	CCT.ATT.TTG.AGG.TAT.TGA.AT GCC.AGT.GTG.CAT.CAC.CA
19.1	40	282	43.43	TGT.TGG.GAT.GCA.AAC.AGG ATC.CCA.CAG.TGC.ATA.AAT

[1]GC clamps:

50 clamp:

CGC.CCG.CCG.CCG.CCC.GCC.GCG.CCC.CGC.GCC.CGT.CCC.GCC.GC
C.CCC.GCC.CG

45 GC clamp:

8

CGC.CCG.CCG.CGC.CCC.GCG.CCC.GTC.CCG.CCG.CCC.CCG.CCC.GG
C.CCG

40 clamp:

CGC.CCG.CCG.CGC.CCC.GCG.CCC.GGC.CCG.CCG.CCC.CCG.CCC.G

8 clamp:

CGT.CCC.GC

5 clamp:

GCG.CG

2 clamp:

CG

[2]Tm is given in %UF

The primer sequences for long and short PCR for the BRCA1 are as

follows:

**Primers for long-PCR BRCA1
(7-PLEX)**

BR1/1-3F   : TgT ACC TTg ATT TCg TAT TCT gAg Agg CTg CTg CTT Ag
BR1/1-3R   : gAg AAA gAA TgA AAT ggA gTT ggA TTT TCg TTC TCA C

Size: **9.9 kb**

BR1/5-9F   : TAg CCA TgA AAA gAT AAT CTC ACA ACT gCC CTT AAg AgC
BR1/5-9R   : ACC AgC CTA CTT gAg ggA ggA Agg Tgg gAA gA

Size: **9.7 kb**

BR1/10-11F   : gAg AgC AgC TTT CAC TAA CTA AAT AAg ATT ggT CAg CTT
                TCT gT
BR1/10-11R   : TCA AgT TTA AgA AgC AgT TCC TTT AAC TAT ACT Tgg AAA
                TTT gT

Size: **4.8 kb**

BR1/12-13F   : gCT Agg ACg TCA TCT TTg gCT TgA ATg AgC TTT A
BR1/12-13R   : gCg ATA ATT ACC CAT gTg CTg AgC AAg gAT CA

Size: **9.0 kb**

BR1/14-17F   : TCT TCA ATg Tgg Agg CAg TAg ggA Tgg AgA AA
BR1/14-17R   : ggg TCT CCA ggT TTT gCC TCA CTT gTT CTT TC

Size: **10.7 kb**

BR1/18-20F   : TCT TAA CTT CAT ATC AgC CTC CCC TAg ACT TCC AAA TAT
                CC
BR1/18-20R   : CAT CTC TgC AAA ggg gAg Tgg AAT ACA gAg Tg

Size: **7.2 kb**

BR1/21-24F   : CAC TCT TCC ATC CCA ACC ACA TAA ATA AgT ATT gTC TC
BR1/21-24R   : gCA TAg CCA gAA gTC CTT TTC Agg CTg ATg TAC AT

Size: **11.4 kb**

```
BC1EX11
Exon Frag Primers 5' -> 3'                              size   Tm(%UF)
   11.1 [GC 3]ACCTTGTTATTTTTGTATATTT  22               347 40.99
        [GC 13]TTGCTAAGCCAGGCTGTT  18

   11.2 [GC 3]ATACTCATGCCAGCTCATTA  20                  461 40.74
        [GC 12]AACGTCCAATACATCAGCTA  20

   11.3       CATGCTCAGAGAATCCTAGA  20                  438 35.04
        [GC 3]CTGTGGCTCAGTAACAAATG  20

   11.4 [GC 12]TCACTCCAAATCAGTAGAGA  20                 476 34.85
        [GC 3]TACTCCTGCTTATAGGTTCA  20

   11.5 [GC 3]GAAAGCAGATTTGGCAGTTC  20                  468 33.66
        [GC 11]CTGACTGGCATTTGGTTGTA  20

   11.6 [GC 3]GAATAGGCTGAGGAGGAAGT  20                  410 40.51
        [GC 13]CTCTTGGAAGGCTAGGATTG  20

   11.7 [GC 3]ACAGCGATACTTTCCCAGAG  20                  345 36.45
             TGCCTTCCCTAGAGTGCTAA  20

   11.8       TTGCAAACTGAAAGATCTGT  20                  365 38.37
        [GC 3]GCTTTGAAACCTTGAATGTA  20

   11.9 [GC 13]GTCGGGAAACAAGCATAGAA  20                 422 40.40
        [GC 4]TTGCCTCTGAACTGAGATGA  20

   11.10 [GC 12]TAATATCACTGCAGGCTTTC  20                292 35.93
         [GC 1]TTCCTCAAAGTTTTCCTCTA  20

   11.11 [GC 1]TCCCATCAAGTCATTTGTTA  20                 390 33.06
              TTCCAGGAAGACTTTGTTTA  20

   11.12 [GC 12]TAATGAAGTGGGCTCCAGTA  20                309 33.22
         [GC 1]CTTCCCATAGGCTGTTCTAA  20

   11.13 [GC 1]GCAAGAATATGAAGAAGTAG  20                 305 37.43
              CAAATGTGTATGGGTGAAAG  20

   11.14 [GC 1]AGACACCTGATGACCTGTTA  20                 378 43.03
         [GC 12]TCTCCTCTGTGTTCTTAGAC  20

   11.15       CCTTTCACCCATACACATTT  20                 460 39.33
         [GC 8]GACTGATGCCTCATTTGTTT  20

   11.16 [GC 3]CTCAGGAACATCACCTTAGT  20                 356 44.00
         [GC 16]ATAAATAGACTGGGCCACAC  20
```

All exons excluding exon 11

Exon Frag Primers 5' -> 3'   size   Tm(%UF)

2   1 [GC 1]TATATATGTTTTTCTAATGTGT 22   203 34.64
      [GC 12]TCCCAAATTAATACACTCTT   20

3   1 [GC 12]GAGCCTCATTTATTTTCT 18   269 37.22
      [GC 4]ATTTTTCGTTCTCACTTA 18

5   1 [GC 4]TATTTGCCTTTTGAGTAT 18   305 26.69
      [GC 12]TCTGATGAATGGTTTTAT 18

6   1 [GC 8]ACTTGCTGAGTGTGTTTC 18   213 35.52
        GCACTTGAGTTGCATTCT 18

7   1 [GC 3]TACATTTTTCTCTAACTGC 19   250 32.67
        GAAGAAAACAAATGGTTTT 19

8   1   GGAGGAAAAGCACAGAAC 18   248 40.51
      [GC 3]CCAGCAATTATTATTAAATACTT 23

9   1 [GC 3]CAGTAGATGCTCAGTAAA 18   242 24.26
        AATACCAGCTTCATAGAC 18

10 1 [GC 4]CTGCATACATGTAACTAG 18   229 38.30
        CTACCCACTCTCTTTTCA 18

12 1 [GC 4]AGTTGCAGCGTTTATAGT 18   289 48.54
      [GC 13]CAGCAAACCTAAGAATGT 18

13 1 [GC 4]GCTTCTCAAAGTATTTCA 18   293 45.18
        AGTGTTTGGCCAACAATA 18

14 1 [GC 4]CCAATTTGTGTATCATAG 18   417 30.78
      [GC 13]AGTGTATAAATGCCTGTA 18

15 1 [GC 1]TGGTTTTCTCCTTCCATTTA 20   303 46.07
      [GC 16]TGTTCCAATACAGCAGATGA 20

16 1 [GC 13]CGTTGTGTAAATTAAACTTC 20   427 47.49
      [GC 1]AGTCATTAGGGAGATACATA 20

17 1 [GC 4]TGTGCTAGAGGTAACTCA 18   242 32.51
      [GC 11]CTCATGTGGTTTTATGCA 18

18 1 [GC 12]TTTCAACTTCTAATCCTTT 19   194 36.32
      [GC 4]GGAGAAATAGTATTATACT 19

19 1   GTTCTTCTGCTGTATGTA 18   178 32.32
      [GC 4]CTGAATGAATATCTCTGG 18

20 1 [GC 4]CTCTTTCTCTTATCCTGAT 19   219 46.40
        TGGTGGGGTGAGATTTTT 18

21 1 [GC 8]ATTCCCCTGTCCCTCTCT 18   172 49.95

CTGGAACTCTGGGGTTCT   18

2 1 [GC 4]TGATTTTACATCTAAATGTC 20   209 47.71
   [GC 13]AGGAGAGAATATTGTGTC 18

3 1 [GC 12]TAGTCCTACTTTGACACT   18   275 49.47
   [GC 4]AAATATTTAAAATGTGCCAA 20

4] [GC 13]AATCTCTGCTTGTGTTCTCT 20   325 59.79
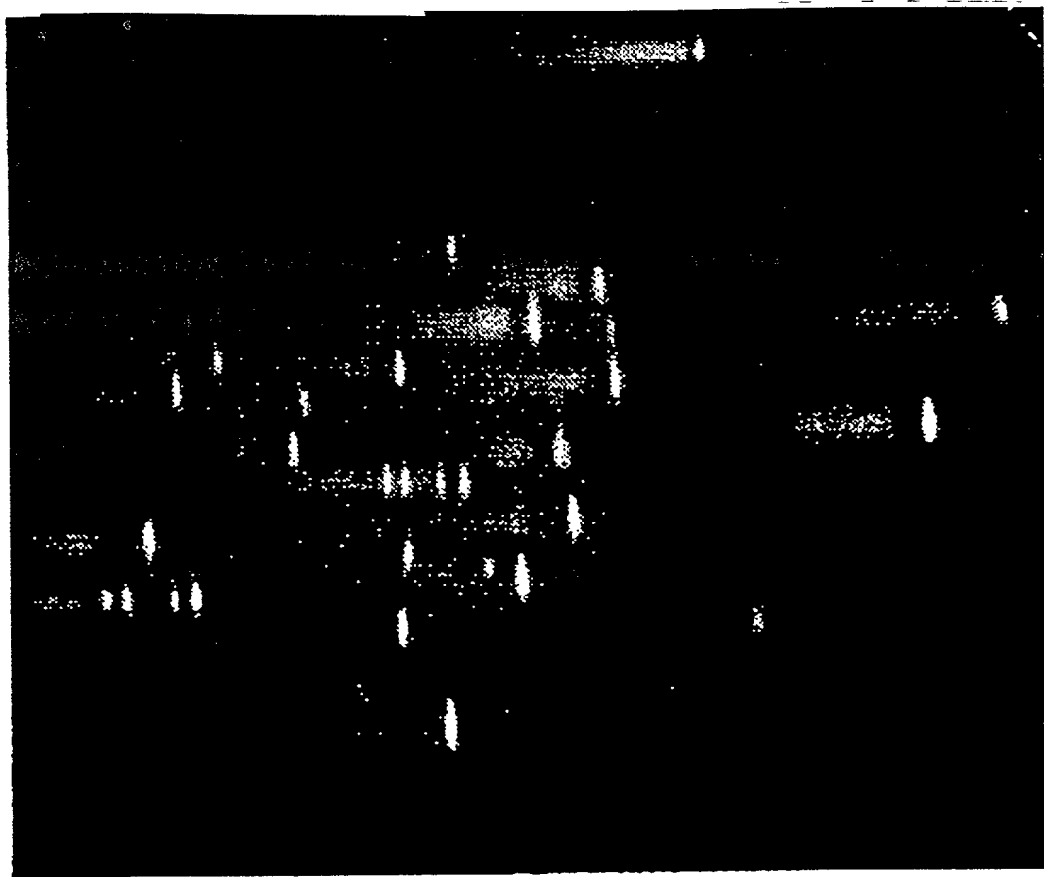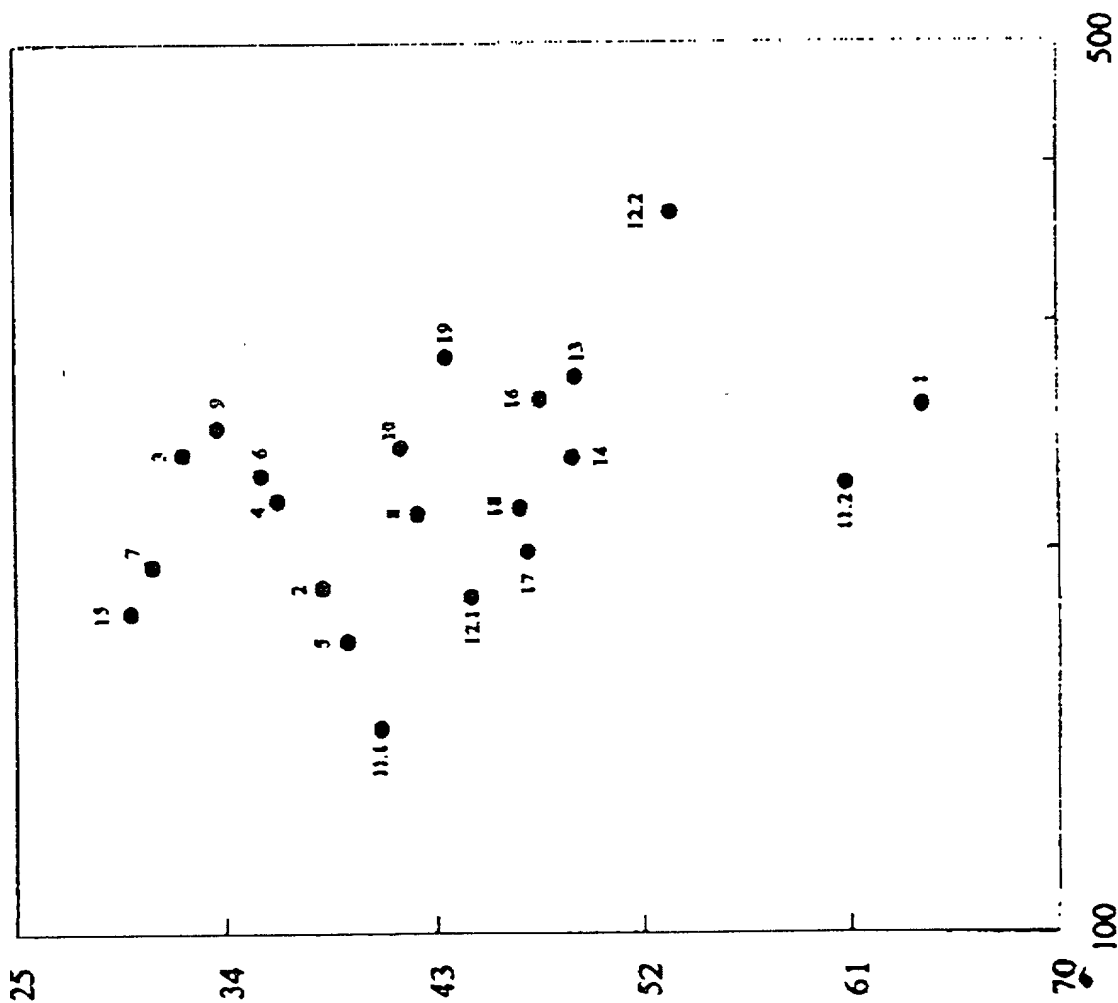   [GC 18]ATTTAGTAGCCAGGACAGTA 20

hMLH1

FIG
1A

BRCA1

FIG. 1B